SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 17, No. 6, pp. 1099–1106, 1998
Printed in the USA
0730-7268/98 $6.00 + .00

RAPID TOXICITY ASSESSMENT OF SEDIMENTS FROM ESTUARINE ECOSYSTEMS: A NEW TANDEM IN VITRO TESTING APPROACH

B. THOMAS JOHNSON*† and EDWARD R. LONG‡
†Environmental and Contaminants Research Center, Biological Resources Division, U.S. Geological Survey, 4200 New Haven Road, Columbia, Missouri 65201
‡Coastal Monitoring and Bioeffects Assessment Division, National Oceanic and Atmospheric Agency, 7600 Sand Point Way NE, Seattle, Washington 98115, USA

(*Received* 17 *January* 1997; *Accepted* 30 *September* 1997)

Abstract—Microtox® and Mutatox® were used to evaluate the acute toxicity and genotoxicity, respectively, of organic sediment extracts from Pensacola Bay and St. Andrew Bay, two estuaries that cover about 273 and 127 km^2, respectively, along the Gulf coast of Florida, USA. The sensitivity and selectivity of these two bioluminescent toxicity assays were demonstrated in validation studies with over 50 pesticides, genotoxins, and industrial pollutants, both as single compounds and in complex mixtures. The 50% effective concentration (EC50) values of insecticides, petroleum products, and polychlorinated biphenyls determined by Microtox all tended to group around the mean EC50 value of 1.2 (0.8) mg/L. The polycyclic aromatic hydrocarbon sensitivity of Mutatox was in general similar to that reported in the Ames test. Surficial sediment samples were collected, extracted with dichloromethane, evaporated and concentrated under nitrogen, dissolved in dimethyl sulfoxide, assayed for acute toxicity and genotoxicity, and compared with reference sediments. Samples with low EC50 values, and determined to be genotoxic, were detected in Massalina Bayou, Watson Bayou, East Bay, and St. Andrew Bay–East in St. Andrew Bay as well as Bayou Grande, Bayou Chico, and Bayou Texar in Pensacola Bay. An overview of these data sets analyzed by Spearman rank correlation showed a significant correlation between acute toxicity and genotoxicity ($p < 0.05$). Microtox and Mutatox in tandem was a sensitive, cost-effective, and rapid (<24 h) screening tool that identified troublesome areas of pollution and assessed the potential sediment toxicity of lipophilic contaminants in aquatic ecosystems.

Keywords—Sediment toxicity Genotoxicity In vitro testing Estuarines Toxicity screening

INTRODUCTION

Coastal bays, estuaries, and wetlands are the conduits of world commerce, playgrounds of the nation, breeding areas and nurseries for fisheries and fowl, and, all too frequently, vast repositories for domestic wastes, agricultural run-off, and industrial chemicals [1]. Toxic anthropogenic and xenobiotic chemical contaminants tend to be lipophilic; as a result, they poorly biodegrade, tend to sorb and accumulate in sediments, and thus potentially move in the aquatic food chain [2–6]. The in situ hazardous nature of these chemical pollutants and their impact on natural resources has created a growing concern as reflected in local, state, federal, and international agencies' efforts to identify and contain, and, in some instances, regulate and remediate sediment pollution [7–9]. As part of the Sediment Toxicity Test Program of the U.S. Geological Survey (USGS) and the National Status and Trends Program of the National Oceanic and Atmospheric Agency (NOAA), this study of the surficial sediments of Pensacola Bay and St. Andrew Bay used two microscale tests—Microtox® and Mutatox® (Microbics, Carlsbad, CA, USA)—to assess the spatial extent and severity of toxicity in the western Florida (USA) panhandle [10–12].

The use of bacterial in vitro assays such as Microtox and Mutatox has become an attractive alternative to traditional and costly fish and invertebrate methods for toxicological screening of sediment [13–28]. Microtox detects acute toxicity (i.e., physiological dysfunctions and lethality) and Mutatox detects genotoxicity (i.e., DNA-damaging potential). The sensitivity of Microtox has been reported by Kaiser and Palabrica [15] in the toxicity data index of over 1,300 organic compounds and by Jacobs et al. [16] in a study of 105 sediment extracts containing Environmental Protection Agency (EPA) semivolatile organic priority pollutants. Microtox, in the basic and solid-phase formats, has been used frequently in studies to detect sediment toxicity; however, the spectrum of sensitivity to complex chemical contaminants in the environment was poorly documented [16–28]. Mutatox, a relatively new assay, has detected genotoxicity in over 100 single chemicals [AZUR Environmental, Carlsbad, CA, USA, unpublished data], as well as in complex mixtures and sediment extracts [29–33]. These two bioluminescent toxicity assays are commercially available.

Microtox and Mutatox were selected for a number of reasons. The protocols are simple and well defined. Media and glassware are standardized and the quantity is minimal, dramatically reducing the disposal cost of toxic waste materials. The assays use freeze-dried test organisms (*Vibrio* formerly *Photobacterium*), which eliminates the tedium and cost of continuous culture; the bacteria are clonal cultures ensuring quality control of the tester strain and diminishing genetic differences. Most importantly, these toxicity bioassays are available on demand and require no preculturing of the test organisms or tedious aseptic techniques. Endpoint measurements of both Microtox and Mutatox are based on bacterial emissions of luminescent light, which simplifies data recovery

* To whom correspondence may be addressed (b_thomas_johnson@nbs.gov).

Use of trade names does not constitute U.S. government endorsement of products.

and reduces instrumentation needs. Data analyses are easily quantified and supported by computer software to implement statistical summation. Data compilations are typically presented in a standard format (i.e., 50% effective concentration [EC50] or genotoxicity designation). Toxicological findings are available in <24 h, frequently in <30 min, whereas traditional fish and invertebrate bioassays require days to complete.

The primary objectives of this investigation were (1) to determine the sensitivity and selectivity of both tests to many different priority organic pollutants [6] and (2) to field test the assays' abilities to assess the potential toxicological hazards of lipophilic chemical contaminants in the sediments of two selected geographic areas. The significance of inorganic and hydrophilic organic toxins in estuarine pollution is recognized but was not considered in this study. These questions were considered: How successful are Microtox and Mutatox in detecting toxicity in complex mixtures? What is the sensitivity and selectivity of these assays to known organic contaminants such as pesticides, polychlorinated biphenyl (PCBs), petroleum products, and polyaromatic hydrocarbons (PAHs) found in sediments, not only as single compounds but also as complex mixtures? Which carrier solvent is the least toxic and most compatible to the test organisms? What is the efficacy of using bacterial tests as broad tier-one screening tools to assess the potential toxicological hazards of lipophilic chemical contaminants in sediments? What is a "toxic" sediment? Do Microtox and Mutatox in tandem assist in "red flagging" or prioritizing specific areas for more comprehensive toxicological profiles?

MATERIALS AND METHODS

Chemicals and biologicals

Pesticides and PCBs were obtained from the Environmental and Contaminants Research Center (ECRC) chemical repository. Polycyclic aromatic hydrocarbons were purchased from Sigma Chemical Company (St. Louis, MO, USA) and Aldrich Chemical Company (Milwaukee, WI, USA). Petroleum products were gifts. All test chemicals were dissolved in acetone and diluted in dimethyl sulfoxide (DMSO), stored frozen in amber bottles with Teflon® cap liners, and thawed at room temperature before use. Solvents were obtained from Sigma and Burdwick Jackson Laboratory (Muskegon, MI, USA). All assay materials for Microtox were purchased from AZUR Environmental (Carlsbad, CA, USA). All test bacteria, media, and glassware for Mutatox were provided through a grant from AZUR Environmental; the rat hepatic S9 fractions used as activation enzymes were obtained from Molecular Toxicology (Annapolis, MD, USA).

Experimental design

Microtox basic toxicity test. Microtox bioassays were conducted according to the standard protocol for the basic test described in the Microtox manual, Volume 3 [34]; glowing luminescent bacteria were exposed to a test substance in a standard four-tube plus control 1:2 dilution series. Samples were incubated at 17°C in a temperature-controlled incubator; light emissions were measured after 5 and 15 min with a luminometer. Phenol and DMSO were the standard positive and negative assay controls; carrier solvent did not exceed 5% of the sample volume. The standard dose–response curve method was used to determine the concentration that caused a 50% loss of light production in the bacteria. Supporting computer software with a standard log-linear model was used to calculate the effective concentration (EC50) values of the test samples. The EC50 values of organic sediment extracts were expressed as mg equivalent sediment wet weight/ml DMSO and reported as the mean value of three replicates performed on separate days using different bacterial reagents; the lower the EC50 value, the higher the acute toxicity of the sample.

Validation of in vitro testing

Sensitivity of the strain of bioluminescent bacteria. Phenol was used with Microtox over an 8-week period to confirm the tester strain's sensitivity over storage time. These data were then compared with the Microtox quality assurance product data accompanying the reagent [34].

Carrier solvent compatibility. The compatibility of carrier solvents used to dissolve lipophilic compounds for Microtox analyses was determined. The positive Microtox phenol control was incubated for 5 min with seven different solvents—acetone, ethanol, methanol, isooctane, hexane, dichloromethane (DCM), and DMSO—at volumes not exceeding 5% of the total assay volume and analyzed with Microtox. These EC50 values were compared with the EC50 values of the positive control to determine which carrier solvent was most compatible, that is, did not significantly change the acute toxicity of phenol.

Mutatox genotoxicity test systems. Mutatox bioassays were conducted according the protocol described by Johnson [29–31]. Nonglowing or dark mutant strains of luminescent bacteria were exposed to a test substance and the amount of light emitted was measured with a luminometer; sample-induced reversion from the nonglowing to glowing phenotype was used to indicate the genotoxicity of the sample. Because most prokaryotic cells, such as the dark mutant strain of *Vibrio,* fail to duplicate vertebrate metabolism of progenotoxins into potential DNA-damaging agents, a mammalian metabolic activation system was incorporated [29–33,35,36]. Test samples were serially diluted in a mixture of bacteria–1% S9 buffer over a 100-fold dose range, incubated in a water bath at 37°C for 15 min in the activation phase, and then incubated at 27°C overnight. Dimethyl sulfoxide, a known compatible carrier solvent with Mutatox [29], was used as the standard solvent in this study. Benzo(*a*)pyrene (BaP) was used as the standard positive control and DMSO was used as the negative control in this assay. A genotoxic response of the luminescent bacteria was determined by measuring the light intensity of each cuvette with a model 500 analyzer. A positive response was defined as a light value of 100 or more and at least three times the light intensity of the bacteria (reagent) control blank. The dose–response number was defined as the number of positive responses recorded at different concentrations per dilution series. A dilution series that contained two or more positive responses at two or more different concentrations was designated "genotoxic"; when the series contained only one positive response, it was designated "suspect"; and when the series contained no positive response, it was designated "negative." Each test sample was determined to be genotoxic, suspect, or negative only after three replicate dilution series were performed on different days.

Single compound and complex mixture sensitivity. Microtox and Mutatox protocols were used to determine the EC50 values and genotoxicity designations of pesticides, PCBs, petroleum products, and PAHs in single compounds and in com-

plex mixtures (see Appendix); carrier solvent did not exceed 5% of either assay's total volume. Complex mixture concentrations were calculated on a volume/volume ratio only.

Environmental sediments

Collection protocol. Forty surficial sediment samples (i.e., the upper 2 cm) from 16 regions in Pensacola Bay and 31 samples from 7 regions in St. Andrew Bay, areas that cover about 400 km^2 along the Gulf coast of Florida, USA, were collected in the spring of 1993 by personnel from NOAA and the Florida Department of Environmental Regulation. Each composited, homogenized sample was packed in ice and shipped by overnight express to ABC Laboratories (Columbia, MO, USA) for organic extraction and finally transferred to the ECRC in Columbia, Missouri, USA, for Microtox and Mutatox analyses. Chemical analyses of samples were conducted by Skidaway Institute of Oceanography (Savannah, GA, USA) [11].

Organic sediment extracts. All sediment samples were processed within 10 d following collection. Samples were warmed to room temperature (20–25°C) and large pebbles, shells, seaweed, wood, crabs, and other such materials were discarded. Excess water was decanted and discarded. The sediment was immediately homogenized with a stainless steel spatula for several minutes. Five-gram samples (wet weight) were removed, dried with anhydrous sodium sulfate, extracted with 100 ml of DCM, carefully evaporated and concentrated under a flow of nitrogen, and brought to a final volume of 1 g sediment wet weight/ml DMSO (mg eq/ml). All organic sediment extracts and controls were dissolved and diluted in DMSO and stored at 5°C in amber bottles with Teflon cap liners until analysis.

Sediment data analysis: Toxicity indices

One could define a toxicant as any agent capable of producing a deleterious response in a biological system, i.e., cellular dysfunction or lethality. This is not, however, a useful working definition for sediment toxicity because virtually every chemical or xenobiotic has the potential to produce injury or death if it is present in a sufficient amount. The Paracelsus principle of "What is there that is not poison? All things are poison and nothing [is] without poison. Solely the dose determines that a thing is not a poison" [37] is the Rosetta stone of modern toxicology. It must be remembered that an EC50 value can be derived for any sediment. The problem is to determine the degree to which a toxicant is poisonous to a resource of interest—in this instance, when a chemical contaminant adversely affects an estuarine sediment. Two concepts were explored to determine when the poison is harmful to the specific resource of interest: the reference comparison and the toxicity index.

The sediment reference is an agricultural soil sample from Florissant, Missouri, USA, with an EC50 value of 49.7 mg eq/ml and no detectable evidence of chemical contamination (i.e., no detectable PCBs, PAHs, pesticides, or metals at <ng/kg sensitivity). To separate endogenous normal background sediment toxicity and present a toxicological reference point, this sediment was spiked with 100 mg/L of phenol. Phenol was selected because it represents a class of commonly found industrial contaminants and has been used by AZUR Environmental for many years as a positive Microtox control. The spiked sediment's Microtox 5 min EC50 value of 5.2 mg eq wet weight sediment extract/ml was given the number one and provided the baseline for the toxicity reference index. Each test sample's EC50 value was compared with this value and placed in the toxicity reference index.

Table 1. Influence of carrier solvents on expression of phenol toxicity (EC50) with Microtox®

Carrier solvent	Acute toxicity EC50 (μg/ml)	Acute toxicity Confidence interval
Phenol	5.5	3.6–8.5
Dimethyl sulfoxide (DMSO) + phenol	6.7	4.5–9.9
Dichloromethane (DCM) + phenol	0.5	0.1–0.9
Hexane + phenol	4.8	3.3–6.8
DMSO + 10% DCM + phenol	10.1	4.0–25.6
Acetone + phenol	21.0	11.6–38.0
Methanol + phenol	8.8	6.1–12.7
95% ethanol + phenol	21.6	13.7–33.9
Isooctane + phenol	6.9	4.6–10.4

The EC50 values were reported as the mean of three replicates with variability expressed as standard deviations. Data sets were tested by analysis of variance (ANOVA) with either a Statistical Analysis System [38] or a Microsoft Excel Analytical ToolPak® software package [39]. Mean differences were measured by one-way ANOVA. If the overall F value was significant ($p \leq 0.05$), the mean differences were ascertained by using the Fisher's least-significant-difference test. Spearman rank correlation was used with GraphPad® software [40] to determine the degree of association between acute toxicity and genotoxicity.

Safety

All extractions and transfers of sediment extracts were conducted under a laminar flow hood to limit the sample's exposure to ultraviolet light and to prevent contamination. Disposable gloves, Tyvek suits, sleeve protectors, and safety glasses were used for handling these potentially hazardous substances.

RESULTS AND DISCUSSION

Bioluminescent bacteria

The positive phenol control detected no changes in the bacteria's sensitivity over an 8-week period. The mean EC50 value for nine analyses (data not shown) of phenol was 15.0 ± 1.9 (±SD) mg/ml, well within the EC50 value range of 13 to 26 mg/ml suggested by AZUR International [34].

Carrier solvent compatibility

Microtox analyses of phenol incubated with seven different carrier solvents clearly demonstrated toxicity deviations (Table 1). Dichloromethane, ethanol, and acetone induced detectable changes in the toxicity of phenol; DCM decreased the EC50 values while ethanol and acetone tended to increased them. Hexane, isooctane, methanol, and DMSO produced no significant changes. Dichloromethane was introduced with DMSO at 1:10 (v/v) and redissolved into acetone:DMSO (1:10, v/v) to simulate possible carryover of the extraction solvent during evaporation; no significant changes in EC50 values were shown. Dimethyl sulfoxide was selected as the carrier solvent because of its low volatility, low freezing point, and low toxicity.

Table 2. Toxicological evaluation of pesticides, polychlorinated biphenyls (PCBs), and petroleum products with Microtox and Mutatox

Test compound	EC50[a]	95% CI[a]	Genotoxicity[b]
Insecticides: Organochlorine			
Aldrin	0.88	0.75–1.05	Negative
Chlorodane (T)	1.3	1.1–1.5	Negative
Dichlorodiphenyldichloroethane	0.82	0.73–0.92	Negative
Dichlorodiphenyldichloroethylene	0.97	0.8–1.2	Negative
Dichlorodiphenyltrichloroethane	1.25	1.04–1.52	Negative
Dieldrin	1.3	1.1–1.7	Negative
Endrin	>50		Negative
Heptachlor	0.95	0.69–1.31	Negative
Hexachlorobenzene	0.63	0.54–0.73	Negative
Kepone	1.41	1.08–1.83	Negative
Lindane	1.56	1.22–1.99	Negative
Methoxychlor	0.86	0.78–0.94	Negative
Mirex	1.2	1.2–1.28	Negative
Pentachlorophenol	0.83	0.77–0.90	Negative
Toxaphene	4.9	2.6–9.5	Negative
Insecticides: Organophosphate			
Dyfonate	2.1	2.0–2.1	Negative
Malathion	0.85	0.64–1.1	Negative
Parathion	0.72	0.68–1.1	Negative
Phorate	3.1	2.6–3.7	Negative
Insecticides: Carbamate			
Carbaryl	0.57	0.52–0.62	Suspect
Carbofuran	0.91	0.74–1.11	Negative
Insecticides: Pyrethroid			
Permethrin	1.56	1.38–1.75	Suspect
Herbicides: Triazine			
Atrazine	3.8	2.9–4.7	Negative
Simazine	4.4	3.3–5.8	Negative
Herbicides: Trifluralin			
Treflan	3.7	2.2–6.3	Negative
Herbicides: Others			
Dacthal	1.3	1.0–1.6	Negative
Industrial: PCBs			
PCB 1242	1.2	0.89–1.6	Negative
PCB 1248	0.55	0.51–0.59	Negative
PCB 1254	1.01	0.87–1.2	Negative
PCB 1254–1260	0.75	0.59–0.95	Negative
Industrial: Others			
Dihexyl phthalate	82	42.2–159.4	Negative
Nonylphenol	0.44	0.29–0.65	Negative
Phenol	15.1	14.2–16.3	Negative
Petroleum products			
Fuel oil #2	0.06	0.04–0.10	Positive
Jet fuel JP4	0.12	0.10–0.13	Positive
Recycled motor oil	1.0	0.82–1.2	Positive
Gasoline	0.16	0.12–0.21	Positive
Crude oil	0.4	0.25–0.64	Positive

[a] Acute toxicity = EC50 = μg/ml, CI = 95% confidence interval, 5-min incubation.
[b] Mutatox® with 1% rat S9 activation.

Validation

Microtox basic test. Microtox detected toxicity in pesticides, PCBs, petroleum products, and PAHs as single compounds and in complex mixtures, all in the mg/L range (Tables 2–4). Different groups of compounds exhibited a number of toxicity patterns. The EC50 values of insecticides, petroleum products, and PCBs all tended to group around the mean EC50 value of 1.2 (0.8) mg/L (Table 2). Herbicides were at least fivefold less toxic than insecticides. In general, the toxicity of PAHs tended to correlate with the ring structure: two-ringed compounds were more toxic than five-ringed compounds (Table 3). For example, the two-ringed PAH acenaphthylene with an EC50 value of 0.34 μg/ml was about 30-fold more toxic than the five-ringed compound 3-methylcholanthrene. Interestingly, pyrene with four rings unsubstituted was not detected at 500 μg/ml. The toxicity of complex mixtures tended to be additive (Table 4) with one significant exception: four- and five-ringed PAHs such as pyrene and BaP tended to be more acutely toxic in complex mixtures containing pesticides and PCBs (Table 4). The two most toxic mixtures were composed

Table 3. Toxicological evaluation of polyaromatic hydrocarbons (PAHs) with Microtox and Mutatox

Compound	EC50[a]	95% CI[a]	Genotoxicity[b]
Acenaphthylene	0.34	0.25–0.47	Positive
Phenanthrene	0.48	0.33–0.68	Positive
Fluorene	0.50	0.35–0.70	Positive
Anthracene	0.64	0.53–0.78	Positive
Benz(*a*)anthracene	0.73	0.65–0.81	Positive
Acenaphthene	0.75	0.69–0.81	Positive
2-Aminoanthracene	0.75	0.49–1.2	Positive
Fluoranthrene	0.83	0.63–1.08	Positive
Naphthalene	0.90	0.85–0.99	Positive
Chrysene	0.92	0.85–0.99	Positive
2-Aminonaphthalene	1.3	1.1–1.52	Positive
2-Acetamidofluorene	2.3	1.26–4.09	Positive
2-Aminofluorene	4.1	2.5–6.4	Positive
Benzo[*a*]pyrene	10.7	6.4–18.2	Positive
3-Methylcholanthrene	19.9	18.3–21.5	Positive
7,12-Dimethyl benzanthracene	33.1	14.6–74.7	Positive
Pyrene	>500		Positive
DMSO (control)	ND[c]		Negative

[a] Acute toxicity = EC50 = μg/ml, CI = 95% confidence interval, 5-min incubation.
[b] Mutatox® with 1% rat S9 activation.
[c] ND = not detected.

of four PCB congeners and four 3- and 4-ringed PAHs (Table 4).

Mutatox genotoxicity assay. The sensitivity of Mutatox to pesticides, PCBs, PAHs, and petroleum products is shown in Tables 3 and 4. Mutatox, more selective than Microtox, detected genotoxicity in single compounds of PAHs and petroleum products but detected no genotoxicity in either pesticides or PCBs at test concentrations of ≤5 μg/ml. Genotoxins were found in complex mixtures of PAHs, petroleum products, PCBs, and pesticides; however, no genotoxins were found in any complex mixture unless PAHs were present (Table 4). All petroleum products were uniformly designated genotoxic. The compounds' sensitivity of Mutatox was in general similar to that reported in the Ames test [41,42].

Table 5. Acute toxicity profile of organic solvent sample extracts[a] from Pensacola Bay and St. Andrew Bay in Florida, USA, with the Microtox

	EC50[b]		Toxicity reference indices	
Location	Mean	SD	Sediment[c]	Phenol[d]
Pensacola Bay[e]	3.61*	3.72	13.8	1.4
St. Andrew Bay[f]	0.88*	1.37	48.8	9.4
Sediment reference[g]	49.7	15.6	1.0	—
Toxicity sediment reference[h]	5.2	0.5	—	1.0

[a] Dichloromethane extraction in DMSO carrier solvent.
[b] * = significant difference from sediment reference ($p < 0.05$), EC50 = mg eq sediment wet weight organic extract/ml.
[c] Sediment toxicity reference index = EC50 value of the Florissant sediment/the EC50 value of the sample.
[d] Phenol toxicity reference index = EC50 value of the phenol-spiked sediment/the EC50 value of the sample.
[e] Pensacola Bay = 40 samples from 16 regions.
[f] St. Andrew Bay = 31 samples from 7 regions.
[g] Sediment reference = an agricultural soil sample from Florissant, Missouri, USA, with an EC50 value of 49.7 mg eq/ml and no detectable evidence of chemical contamination (i.e., no PCBs, PAHs, pesticides, or metals).
[h] Toxicity sediment reference = sediment reference spiked with 100 mg/L of phenol.

Sediment toxicity

Organic extracts of sediment samples were designated "toxic" when their EC50 values were significantly less than the EC50 value of 5.2 (0.5) mg eq sediment extract/ml (Table 5) of the sediment reference.

Microtox analysis: Acute toxicity. Organic extracts of 71 sediment samples from 23 regions in St. Andrew Bay and Pensacola Bay showed evidence of acutely toxic substances. A composite profile of all samples ($n = 71$) from both bays showed toxicity in 63 of 71 samples (Table 5). The mean EC50 value was 0.88 mg eq sediment extract/ml for all St. Andrew Bay samples and 3.6 for Pensacola Bay. These findings sug-

Table 4. Toxicological evaluation of complex mixtures containing PCBs, PAHs, and pesticides with Microtox

Complex mixture[a]	EC50[b]	95% CI[b]	Genotoxicity[c]
PCBs: 1242 + 1248 + 1254 + 1260	0.9	0.8–0.9	Negative
DDT + DDE + DDD	1.5	1.3–1.7	Negative
Kepone + aldrin + lindane + DDT + PCB1254	1.6	1.4–1.7	Negative
Phenanthrene + chrysene + anthracene + benzo(*a*)pyrene	0.6	0.6–0.7	Positive
Aminoanthracene + benzo(*a*)pyrene + aminofluorene + 3-methylcholine	3	1.6–2.0	Positive
Aminoanthracene + benzo(*a*)pyrene + aldrin + DDT	1.8	1.6–2.0	Positive
Aldrin + DDT + heptachlor + endrin	1.6	1.1–2.2	Negative
Altrazine + DDT + aldrin + PCB 1254 + pyrene	1.7	1.4–2.1	Positive
DDT + benzo(*a*)pyrene + PCB 1254 + 1260 + atrazine	2.2	1.6–2.9	Positive
Carbofuran + carbaryl + atrazine + treflan	1.7	1.4–2.1	Negative
Carbofuran + carbaryl + atrazine + permethrin	1.2	0.9–1.5	Negative
Carbofuran + DDT + atrazine + permethrin	1.6	1.5–1.6	Negative

[a] Complex moisture = v/v.
[b] Acute toxicity = EC50 = μg/ml, CI = 95% confidence interval, 5-min incubation.
[c] Mutatox® with 1% rat S9 activation.

Table 6. Sediment toxicity of St. Andrew Bay in Florida, USA, with Microtox® and Mutatox®

Sample[a]		EC50[b]		Toxicity reference indices		Genotoxicity[c]
Region	Location	Mean	SD	Sediment[d]	Phenol[e]	Designation
A	West Bay	2.13*	1.73	23.3	2.4	Negative
B	North Bay	0.72*	0.58	69.0	7.2	Positive/negative
C	St. Andrew Bay–West	0.92*	0.84	54.0	5.7	Positive
D	Massalina Bayou	0.17*	0.04	292.4	30.6	Positive
E	Watson Bayou	0.43*	0.31	115.6	12.1	Positive
F	St. Andrew Bay–East	1.60*	2.79	31.1	3.3	Positive
G	East Bay	0.49*	0.19	101.4	10.6	Negative
Control	Sediment reference[f]	49.5	3.95	1.0	—	Negative
Control	Toxicity sediment reference[g]	5.2	0.5	—	1.0	Negative

[a] Dichloromethane extinction in DMSO carrier solvent.
[b] * = significant difference from sediment reference ($p < 0.05$), EC50 = mg eq sediment wet weight organic extract/ml.
[c] Rat hepatic S9 activation.
[d] Sediment toxicity reference index = EC50 value of the Florissant sediment (49.5 mg eq sediment/ml)/the EC50 value of the sample.
[e] Phenol toxicity reference index = EC50 value of the phenol-spiked sediment (5.2 [0.5] mg eq sediment/ml)/the EC50 value of the sample.
[f] Sediment reference = an agricultural soil sample from Florissant, Missouri, USA, with an EC50 value of 49.7 mg eq/ml and no detectable evidence of chemical contamination (i.e., PCBs, PAHs, pesticides, or metals).
[g] Toxicity sediment reference = the sediment reference spiked with 100 mg/L of phenol.

gested that sediments from St. Andrew Bay and Pensacola Bay were about 50-fold and 14-fold more toxic than the sediment reference; the toxicity of St. Andrew Bay was about fourfold greater than that of Pensacola Bay. Regions were designated acutely toxic when the toxicity reference index numbers were greater than 1. For example, the Bayou Grande region had a toxicity reference index number of 14.1 (phenol-spiked sediment reference's EC50 value/test sample's EC50 value = toxicity reference index number; 5.2/0.37 = 14.1), indicating that this sample was about 14-fold more acutely toxic than the phenol-spiked sediment. Sediments in Massalina Bayou, Watson Bayou, East Bay, and St. Andrew Bay–West in St. Andrew Bay, as well as Bayou Grande, Bayou Chico, and Bayou Texar in Pensacola Bay had toxicity reference index numbers >5 (Tables 6 and 7) and were designated acutely toxic. The toxicity reference index numbers of 9.4 for St. Andrew Bay and 1.4 for Pensacola Bay indicated both bays were more toxic than the phenol-spiked sediment; whereas St. Andrew Bay was

Table 7. Sediment toxicity profile of Pensacola Bay in Florida, USA with Microtox® and Mutatox®

Sample[a]		EC50[b]		Toxicity reference indices		Genotoxicity[c]
Region	Location	Mean	SD	Sediment[d]	Phenol[e]	Designation
A	Bayou Grande	0.37*	0.35	133.4	14.1	Positive/suspect
B	Bayou Chico	0.48*	0.21	103.1	10.8	Positive
C	Bayou Texar	0.68*	0.47	72.8	7.6	Positive/negative
D	Warrington	7.29*	1.92	6.8	0.7	Negative
E	Bayou Channel	4.72*	1.65	10.5	1.1	Suspect/negative
F	Inner Harbor	1.97*	1.92	25.2	2.6	Positive
G	Harbor Channel	10.48*	0.19	4.7	0.5	Negative
H	Lower Bay	10.36*	1.60	4.8	0.5	Suspect/negative
I	Central Bay	1.84*	0.25	26.9	2.8	Positive
J	East Bay	1.11*	0.24	44.6	4.7	Negative
K	East Bay extension	2.49*	1.23	19.9	2.1	Negative
L	Blackwater Bay	3.25*	4.14	15.3	1.6	Suspect/negative
M	Escambia Bay	4.72*	1.02	10.5	1.1	Suspect/negative
N	I-70	1.46*	1.15	33.9	3.6	Suspect
O	River Delta	6.75*	3.04	7.3	0.8	Positive/suspect/negative
P	Floridatown	3.39*	1.56	14.6	1.5	Positive/suspect
Control	Sediment reference[f]	49.5	3.95	1.0	—	Negative
Control	Toxicity sediment reference[g]	5.2	0.5	—	1.0	Negative

[a] Dichloromethane extraction in DMSO carrier solvent.
[b] * = significant difference from reference sediment ($p < 0.05$), EC50 = mg eq sediment wet weight organic extract/ml.
[c] Rat hepatic S9 activation.
[d] Sediment toxicity reference index = EC50 value of the Florissant sediment (49.5 mg eq sediment/ml)/the EC50 value of the sample.
[e] Phenol toxicity reference index = EC50 value of the phenol-spiked sediment (5.2 [0.5] mg eq sediment/ml)/the EC50 value of the sample.
[f] Sediment reference = an agricultural soil sample from Florissant, Missouri, USA, with an EC50 value of 49.7 mg eq/ml and no detectable evidence of chemical contamination (i.e., PCBs, PAHs, pesticides, or metals).
[g] Toxicity sediment reference = the sediment reference spiked with 100 mg/L of phenol.

nearly 10-fold more toxic, Pensacola Bay was only slightly more toxic. The coefficient of variation of samples within a single region was about 17%, quite good for bioassays. The reader will note that the standard deviations of composite regions were large as would be expected considering that the samples were taken from such vast areas, frequently kilometers apart. Toxicological trends, however, can be seen because significant toxicological variations between different regions (Tables 6 and 7) clearly identified areas of concern.

Mutatox analysis. Six regions in Pensacola Bay were classified genotoxic positive, six suspect, and only four negative. Five regions in St. Andrew Bay were classified positive; only two regions with no evidence of genotoxins—West Bay and East Bay—were designated negative. About 40% of the 71 organic extracts of sediment samples analyzed with the rat-activated Mutatox showed evidence of DNA-damaging substances and were designated genotoxic (Tables 6 and 7); 14% were classified as suspect. These data suggested that genotoxins were evident and widespread in sediment residues from both bays.

Tandem testing. Gregus and Klaassen [37] have noted that measures of acute lethality such as the EC50 value may not accurately reflect the spectrum of toxicity, or the hazard, associated with exposure to a chemical contaminant. For instance, a chemical contaminant may have carcinogenic or mutagenic effects at doses that produce no evidence of acute toxicity to resources of interest. Microtox and Mutatox tests of PAHs are illustrative (Table 3). For example, acenaphthene, acenaphthylene, and fluorene produced low 5-min EC50 values of less than 1 μg/ml and were considered acutely toxic. On the other hand, BaP and pyrene produced 5-min EC50 values greater than 10 μg/ml (far exceeding their water solubility) and could be considered low-risk aquatic contaminants. However all five PAHs tested as genotoxic and are potentially carcinogenic agents. Pyrene and BaP confirmed this pattern described by Gregus and Klaassen [37].

Tandem use of acute toxicity and genotoxicity screening tests significantly broadened the scope of environmental risk assessment. Microtox and Mutatox as sister assays were ideally suited for tandem testing, simple to perform, rapid with data available in less than 24 h, and sensitive to a broad spectrum of known organic chemical contaminants. Pensacola Bay and St. Andrew Bay data sets produced by Microtox and Mutatox and analyzed with Spearman rank correlation showed a significant correlation of acute toxicity and genotoxicity ($p \leq 0.001$). Samples from Bayou Grande, Bayou Chico, and Bayou Texar in Pensacola Bay as well as from Massalina Bayou, Watson Bayou, East Bay, and St. Andrew Bay–East in St. Andrew Bay were designated acutely toxic and genotoxic. These regions were identified as areas of concern and warranted additional toxicological characterizations. Preliminary chemical analysis of these sediment extracts revealed pesticide, PCB, and PAH residues [11] that suggested a correlation with acute toxicity and genotoxicity data. Release of these findings is planned in a future publication.

SUMMARY

These two in vitro bioassays rapidly determined both the acute toxicity and genotoxicity of organic sediment extracts from two large estuarine ecosystems in Florida. Sediment toxicity protocols for Microtox and Mutatox with lipophilic organic contaminants were established. The sensitivity and selectivity of Microtox and Mutatox tests with over 50 chemical contaminants of interest that included PCBs, pesticides, and PAHs as single compounds and in complex mixtures were validated. Toxicity indices to define hazardous lipophilic chemical contaminants in sediment samples were developed. Tandem use of an acute toxicity test and a genotoxicity test as a simple, rapid risk assessment screening tool to identify specific toxicological areas of concern was demonstrated.

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APPENDIX

Validation: Environmental chemical contaminants frequently recovered from sediment

INSECTICIDES			
Organochlorine			
Aldrin	Chlordane	Heptachlor	DDD
Kepone	Hexachlorobenzene		DDE
Toxaphene	Lindane	DDT	
Methoxychlor	Dieldrin	Mirex	
Endrin	Pentachlorophenol		
Organophosphate			
Malathion	Parathion	Dyfonate	
Carbamate			
Carbaryl	Carbofuran	Phorate	
Pyrethroid			
Permethrin			
HERBICIDES			
Triazine			
Atrazine	Simazine		
Trifluralin			
Treflan			
Others			
Dacthal			
INDUSTRIAL PRODUCTS			
Polychlorinated biphenyls			
1242, 1248, 1254, 1260			
Phthalates			
Dihexyl phthalate			
Phenols			
Phenol	Nonylphenol		
PETROLEUM PRODUCTS			
Gasoline	Jet fuel (JP4)	Recycled motor oil	
Fuel oil #2	Crude oil (Amoco)		

POLYAROMATIC HYDROCARBONS	
Acenaphthene	Chrysene
Acenaphthylene	Dibenz(*a,h*)anthracene
2-Acetamidofluorene	7,12-Dimethyl benzanthracene
2-Aminoanthracene	Fluoranthrene
2-Aminonaphthalene	Fluorene
Anthracene	Indeno(1,2,3-*c,d*)pyrene
Benz(*a*)anthracene	3-Methylcholanthrene
Benzo(*a*)pyrene	Naphthalene
Benzo(*b*)fluoranthene	Phenanthrene
Benzo(*ghi*)perylene	Pyrene